Regulation of Uterine and Ovarian Gene Function by Chlorpyrifos and Dimethoate Exposure in Mammalian Model

PhD Synopsis

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1. Background

The use of pesticides is almost as old as agriculture. Although the use of these chemicals has been of great benefit to human life, increasing evidence has demonstrated several negative effects on the environment and human health. The exposure to pesticides has been associated with the incidence of different chronic diseases such as cancer, birth defects, reproductive disorders, diabetes, cardiovascular diseases, and central nervous system pathologies, like Parkinson, Alzheimer or Amyotrophic Lateral Sclerosis (ALS) (Mostafalou & Abdollahi, 2013). Chlorpyrifos and dimethoate are the organophosphate insecticides extensively used in agricultural and domestic settings. Chlorpyrifos has been found to cause some developmental defects in rats (Adigun et al., 2010). Dimethoate has been reported to cause developmental toxicity as well as reproductive failures in organisms upon repeated exposures (Okoroiwu & Iwara, 2018). These pesticides can act as endocrine disrupting chemicals (EDCs), able to interfere in the synthesis, secretion, transport, binding, action or elimination of natural hormones, responsible for the maintenance of homeostasis of events such as reproduction and development (Zoeller et al., 2012). Female reproductive lifespan is largely determined by the size and the quality of the primordial follicle pool established early in life (the ovarian reserve) (Jirge, 2016). A dynamic decline in this pool occurs within the human ovary with age, culminating in the natural menopause at age 50 ± 4 years (Depmann et al., 2015). However, it has been estimated that large numbers of women worldwide are suffering from early menopause (EM) or experience primary ovarian insufficiency (POI), 12.2% and 3.7% respectively (Golezar et al., 2019). Furthermore, certain maternal and environmental

factors can disrupt oogenesis contributing to compromised oocyte quality and leading to arrested development, reduced fertility and epigenetic defects that affect offspring long-term health (Mtango et al., 2008; Plante et al., 2022; Delbes et al., 2022). An increase of 1.45% from 2007 to 2017 in global age-standardized incidence rate for polycystic ovarian syndrome (PCOS) (Liu et al., 2021) was reported, which affects 5% - 20% of women of reproductive age (Azziz et al., 2016). Furthermore, ovulation disorders like PCOS and POI, are estimated to account for infertility in about 1 of 4 infertile couples (Duursen et al., 2020). Endometriosis and uterine fibroids (UFs), often present as comorbidities in diseased patients, are further contributing to women's infertility (Uimari et al., 2011). Found in up to 80–90% of women at the age of 50 (Parker, 2007; Grube et al., 2019), UF are associated with increased risk of pregnancy loss and obstetric complications and may be the sole cause of infertility in 2–3% of women (Freytag et al., 2021). A growing body of evidence in the last few decades implicates EDCs in the aetiology of reproductive pathologies (Diamanti-Kandarakis et al., 2009). Precise endocrine signalling is involved in all aspects of the development and functioning of the female reproductive system. Indeed, despite their multifactorial nature, estrogens seem to play a central role in disease pathogenesis of endometriosis and UFs (Borahay et al., 2017; Upson, 2020). In addition, overexposure to androgens in utero is thought to be responsible for PCOS development later in life (Filippou and Homburg, 2017) and can also affect among others, the early establishment of ovarian reserve (Richardson et al., 2014; Johansson et al., 2020). The direct consequences of the detrimental effects of EDCs on female reproductive health are impaired reproductive organ function, infertility and cancer. However ovarian dysfunction can lead to reduced serum estradiol levels, which are associated with increased risk of cardiovascular diseases (Jeon et al., 2010), loss of bone density

(Rannevik etal., 1995; Cummings et al., 1998), and sexual dysfunction (van der Stege et al., 2008). Exposure to environmental factors, mainly nutritional, chemical and physical factors, potentially alters gene expression and changes the epigenome that can modify adult disease susceptibility and lead to disease phenotype (Axelsson et al., 2010; Dada et al., 2012). The gene-environment interaction may be one of the major contributors to female infertility, and have lifelong (Macon & Fenton, 2013) and transgenerational impact (Newbold et al., 2006). Hence, studying the expression levels and epigenetic modifications (methylation profile) of key development-related and functional genes of uterus and the ovary following the pesticide exposure (chlorpyrifos and dimethoate) will elucidate the effect of pesticide exposure on the critical and sensitive windows of female reproduction and fertility. Therefore, the extreme sensitivity of the female reproductive system to disruption by chemicals that may interfere with estrogen and androgen synthesis and actions, together with the timing of the exposure are important considerations for the later outcome (Fowler et al., 2012).

The presence and activity of Wingless-type mouse mammary tumor virus integration site (WNT) signaling components in the ovary is not unexpected given the variety of physiological processes known to be regulated by the WNT family of proteins. WNT signaling is classified broadly into canonical [β-catenin (CTNNB1)- dependent, also referred to as WNT/β-catenin signaling] and non-canonical (CTNNB1-independent) pathways. A series of studies have identified the expression and regulation of WNT ligands and downstream WNT signaling components in the developing follicle and corpus luteum of rats, mice, humans, and cattle (Harwood et al. 2008; Castanon et al. 2012; Gupta et al. 2014). However, the epigenetic regulation of WNT signaling

(canonical and non-canonical) has not been unveiled fully owing to the contribution related to female reproductive health.

Following this line of reasoning, the study is designed to study the impact of chlorpyrifos and dimethoate exposure on the expression of uterine genes (HOXA10, HOXA11, WNT5A) responsible for uterine arrangement and differentiation and ovarian genes (ESR2, RSPO2) having role in follicular development and maturation. Further, the study will identify the methylation profile of these genes following the pesticide exposure and will investigate the molecular implications of chlorpyrifos and dimethoate exposure in regulating the WNT signaling in uterus and ovaries of female Wistar rats.

2. Aim

To study the impact of chlorpyrifos and dimethoate exposure on the expression and methylation profile of uterine (HOXA10, HOXA11, WNT5A) and ovarian (ESR2, RSPO2) genes regulating the reproductive health and thereby fertility in females.

3. Objectives

1) To investigate the effect of chlorpyrifos and dimethoate exposure on expression of uterine (HOXA10, HOXA11, WNT5A) and ovarian (ESR2, RSPO2) genes in female Wistar rats.
2) To identify the methylation profile of uterine (HOXA10, HOXA11, WNT5A) and ovarian (ESR2, RSPO2) genes following the exposure to chlorpyrifos and dimethoate.
3) To investigate the molecular implications of chlorpyrifos and dimethoate exposure on regulation of WNT signaling in uterus and ovaries of female Wistar rats.

4. Review of Literature

A female's reproductive lifespan depends on the size and health of the initial pool of primordial follicles and their progression and maturation into primary, secondary, antral, and eventually ovulatory follicles. Complex bidirectional communication occurs between the oocyte and its surrounding somatic cells involving stimulatory inputs from local paracrine factors as well as steroid hormones (Kezele & Skinner, 2003; Skinner, 2005; Chen et al., 2007). The gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), have a significant role in the selection and maturation of the follicles via stimulation of IGF-1 and estrogen signaling pathways among others (Richards et al., 2002). Once an oocyte is fertilized, the implantation of the embryo into the uterus and successful pregnancy and parturition are dependent on healthy uterine function. These processes are regulated by the WNT and HOX gene families and are responsive to IGF-1 and estrogen signaling as well (Daftary & Taylor, 2006; Hewitt et al., 2009; van der Horst et al., 2012). Therefore, EDC exposure may have the potential to reprogram ovarian and uterine functions.

Pesticides can act as EDCs. Pesticides are mainly used

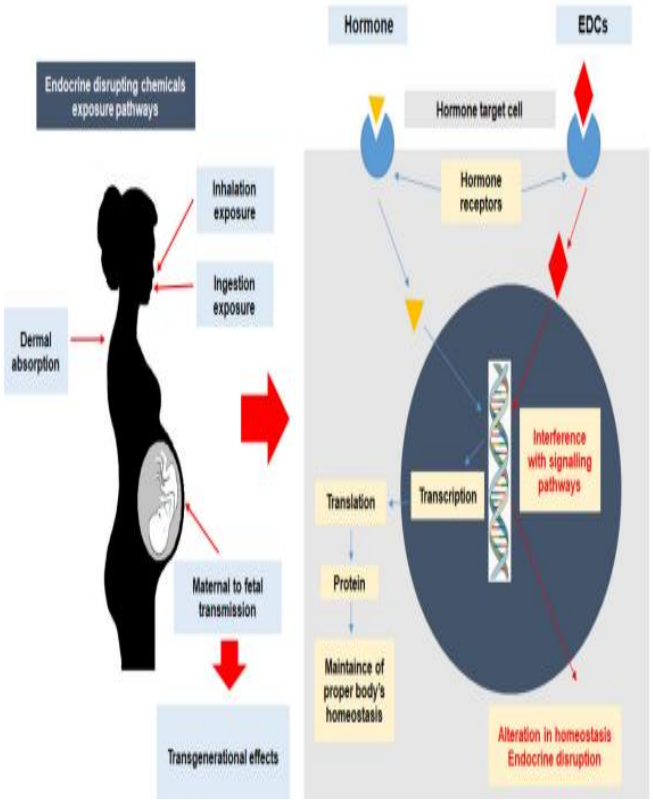

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Fig. 1 Different exposure pathways of EDCs to women and their effect on hormone mediated homeostasis

in agricultural practice, to increase crop productivity, but other applications include home gardens, handling with domestic animals by the use of dewormers, or even to control infectious diseases. So, human exposure to pesticides can occur through many routes, like occupational dealing with production, transport, delivery and application of pesticides themselves, residing in places with high incidence of pesticide residues, as well as circulation and accumulation of these chemicals in the food chain (Mostafalou & Abdollahi, 2017). Depending on the amount of pesticides used, these can accumulate in plants, water, soil, air and biota, meaning they can contaminate soil and water, remain in crops, and finally enter the food chain, thereby posing a threat to humans (Sharma et al., 2019). Indeed, fruits and vegetables, which are strongly encouraged to be consumed given their richness in essential nutrients, not only to prevent vitamin deficiency, but also to reduce the incidence of certain pathologies, such as cancer, cardiovascular diseases, obesity, among others, are often the top of contaminated food (Nassan et al., 2018). In this way, diet is the main source of exposure to pesticides, the so-called chronic exposure, for the majority of the worldwide population, and a concerning fact resides in the limited knowledge on the chronic adverse effects of low doses, compared to the knowledge on the acute toxic effects observed in humans, upon sudden exposure to high doses of pesticides (Marques-Pinto & Carvalho 2013; Martín Reina et al., 2017). Moreover, the presence of pesticide residues and/or their degradation products can even be more toxic than their precursors (Morais et al., 2012). Blood disorders (anaemia, defective blood coagulation), brain and nerve damage, paralysis, jaundice, hepatic fibrosis, allergenic sensitization, emphysema, asthma, kidney problems, cancer, genetic disorders, birth defects, miscarriage, impotence, and infertility or sterility are variously associated with chronic exposure and low residual level of pesticides (Gold et al., 2001).

Chlorpyrifos (CPF) [O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate] is a broad spectrum organophosphate (OP) insecticide used for a variety of agricultural and domestic applications (EPA, 2008). It is the fourth highest consumed insecticide in India and residues have been detected at a mean level of 0.0662 mg/L in human blood, 1.45 mg/kg in eggplant, 0.092 lg/ mL in milk samples from cow, buffalo, doe and ewe (Mathur et al., 2005; Baig et al., 2009; Karabasanavar and Singh, 2012). A decreased body weight, appetite, milk production and interrupted estrous cycle have been found to be associated with continuous low level exposure to pesticides in human, laboratory and farm animals (Tiemann, 2008). Reproductive behaviour is considered as a promising tool in ecotoxicology which provides information about the biochemical, physiological and toxicological reactions to a chemical related to reproduction (El-Kashoury and El-Din, 2010; Attia et al., 2012) and many of the tested pesticides have been found to be more toxic to females than males of a species (Mancini et al., 2005). Chlorpyrifos has been found to cause some developmental defects when exposed orally in rats (Adigun et al., 2010). Dimethoate [(0, 0-dimethyl-S (N-methylcarbonyl methyl) phosphorodithioate)] is another organophosphate that has been reported to cause developmental toxicity as well as reproductive failures in organisms upon repeated exposures (Okoroiwu & Iwara, 2018). Developmental toxicity of dimethoate includes decreased number of implantations and live fetuses, incidences of resorptions and decreased fetal body weights (Farag et al., 2007). Reproductive toxicity of this pesticide on adult rodents of both sexes have been demonstrated. Irregularities of estrous cycle and altered level of serum gonadotrophins have been reported in females (Mahadevaswami & Kaliwal, 2002; Kaur & Dhanju, 2005). While, impairment of fertility, suppressed libido, semen

quality deterioration, altered testosterone levels and testicular degeneration are few of the reports available in males (Ngoulaa et al., 2007; Louis et al., 2015).

There are numerous lines of evidence emerging that suggest that the exposure to estradiol or estrogenic EDCs can cause epigenetic alterations in sensitive developmental windows that might have long-term effects by adulthood (Jefferson et al., 2013; Zama & Uzumcu, 2013).

Hence, genetic and epigenetic studies are needed to deduce the mechanistic approach of the EDCs. The study aims to investigate the extent of chlorpyrifos and dimethoate exposure on the expression and methylation profile of uterine (HOXA10, HOXA11, WNT5A) and ovarian (ESR2, RSPO2) genes.

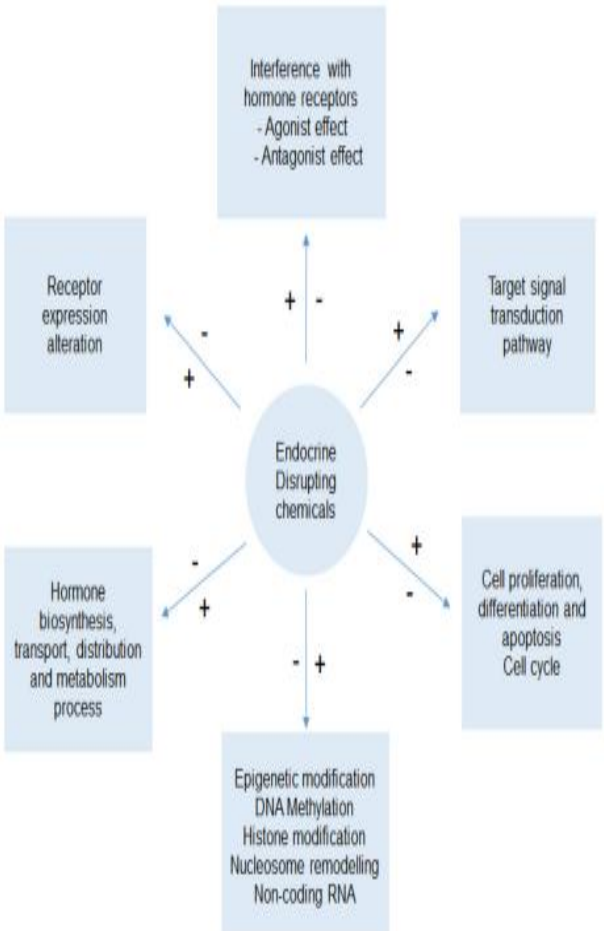

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Fig. 2 Mode of actions of endocrine disruptors via different targets.

Homeobox genes (HOX genes) comprise a group of highly conserved genes that are essential regulators of anterior–posterior axial pattern development. Female fertility is a broad term, which includes the ability to reproduce or become pregnant. Multiple factors influence female fertility, including normal aging and several disease processes. However, two processes are essential for normal female fertility: ovarian

follicular maturation and embryo implantation. In vertebrates, HOX genes are involved in both of these processes. The overlapping expression patterns of HOXA/Hoxa10 and HOXA/ Hoxa11 make both these genes the requisites for uterine arrangement and differentiation, including the functional differentiation of the endometrium (Taylor, 2000; Daftary & Taylor, 2006; Du & Taylor, 2016). However, HOXA/Hoxa10 was the first gene that has emerged as a promising marker of fertility due mainly to its role in altering the adult uterine environment (Taylor, 2000; Daftary & Taylor, 2006; Modi & Godbole, 2009). HOXA/Hoxa10 is expressed in the luminal and glandular epithelium and stroma of the endometrium as well as the myometrium. Since HOXA/Hoxa10 is expressed in adult murine, canine, primate, human, and porcine uteri, the conservation and versatility of the Hoxa10 gene has been an area of interest regarding fertility outcomes in mammalian females (Blitek et al., 2010; Wang et al., 2019). HOXA/Hoxa10 mutations or altered expression result in subfertility or infertility (Connell et al., 2013; Du & Taylor, 2016). In addition, variations in HOXA10 gene expression have been observed during several reproductive anomalies, including endometriosis, polycystic ovarian syndrome (PCOS), leiomyoma, polyps, adenomyosis, and hydrosalpinx (Connell et al., 2013).

Non-canonical WNTs, including WNT5a and WNT11, are expressed in granulosa cells (GCs) and are differentially regulated throughout follicle development. Wnt5a and Wnt11 expression has been reported in mouse granulosa cells (GCs) (Harwood et al., 2008), and Wnt5a transcripts have been detected in human luteinized GCs and cumulus cells (Kenigsberg et al., 2009; Sanchez et al., 2014). Expression of Wnt5a has been shown to be down-regulated by FSH in a bovine primary GC culture model (Abedini et al., 2015).

Estrogen action is mediated by two nuclear receptors, estrogen receptors alpha (ERα) and beta (ERβ), encoded by the Esr1 and Esr2 genes in mice. These receptors bind their cognate ligands e.g. 17β-estradiol (E2) and other estrogenic compounds and regulate transcription by binding estrogen response elements in the genome or through interactions with other transcription factor complexes. E2 plays an important role in both male and female fertility, and in females E2 signaling has several essential roles including regulation of the estrous cycle and uterus function. The generation of mice with disrupted Esr1 or Esr2 genes has illustrated the importance of both these genes in reproduction. Disruption of the Esr1 gene causes infertility in both male and female mice. Female ERα knockout (KO) mice have increased serum E2 levels and no estrous cycle, a hypoplastic uterus, and hemorrhagic cysts in the ovaries (Lubahn et al., 1993; Dupont et al., 2000; Chen et al., 2009). Conditional knockouts with deletion of ERα in neurons or pituitary have been shown to result in infertility (Wintermantel et al., 2006). ERβ has a more restricted expression pattern than ERα with follicular granulosa cells in the ovary displaying the highest expression (Cheng et al., 2002). Consequently, female mice with disrupted Esr2 have a less severe reproductive phenotype, but different phenotypes have been reported ranging from reduced fertility to infertility in females and normal fertility to infertility in males (Krege et al., 1998; Dupont et al., 2000).

R-spondins (RSPO) are secreted proteins that promote the activation of the canonical WNT/β-catenin (CTNNB1) signaling pathway. R-spondins can stimulate cell proliferation by potentiating WNT/CTNNB1 signaling (Kim et al., 2005). During development, R-spondin1 (Rspo1) is required for ovarian differentiation and is downregulated in the postnatal ovary (Parma et al., 2006; Chassot et al., 2008). In contrast, the expression of R-spondin2 (Rspo2) has been detected in mice oocytes at

birth and Rspo2 heterozygous loss-of function female mice have been shown to gradually lose their fertility from 4 months of age (Bell et al., 2003; Nam et al., 2007).

WNTs are highly conserved signaling molecules that act through β-catenin dependent and β-catenin independent pathways to regulate important processes of cellular growth and differentiation including cell proliferation, cell fate specifications, embryonic induction and the generation of cell polarity (Miller et al. 1999; Komiya & Habas 2008). Misregulation of WNT signal transduction can lead to a variety of pathologies including development of carcinomas of the breast, colon, skin, and ovary (Polakis 2000; Giles et al., 2003; Logan & Nusse 2004, Boerboom et al., 2005). The foundational study establishing a requirement of WNT signaling molecules in the female ovary was performed by Vainio et al. (1999). This group utilized mice null for Wnt4 to demonstrate a role for this molecule in early ovarian development and suppression of the male reproductive tract. Wnt4 null females had sex-reversed ovaries that expressed genes associated with testicular development, along with a reduced number of oocytes at birth. Subsequent work aimed at elucidating the importance of WNT signaling in the postnatal ovary has identified multiple Wnt/WNT family member transcripts expressed at specific stages of follicle development within the adult ovary of mice, rats, humans and cattle (Hsieh et al., 2002; Ricken et al., 2002; Wang et al., 2009; Gupta et al. 2014). In addition, functional studies in the adult ovary have shown a fundamental requirement of WNT signaling for normal ovarian function and fertility. Though the understanding of contributions of WNT signaling to the regulation of folliculogenesis has grown tremendously in recent years, much still remains unknown about the broader physiological involvement of WNT signaling in the adult ovary.

Keeping in view these findings, it is conceivable to study the uterine and ovarian gene expression and methylation profile following the chlorpyrifos and dimethoate exposure in female rats to identify the effect of pesticide exposure on the critical and sensitive windows of female reproduction and fertility and the epigenetic players which could influence the WNT signaling cascade via modulation of its components and coordinated regulation of the WNT target genes.

5. Identification of Research Gaps

Understanding the impacts of chemical exposures on reproductive success in females is a daunting task. It is complicated by the heterogeneity of the factors contributing to the reproductive dysfunction, varying knowledge of the endocrine control of reproductive development and/or the ease at which reproductive success can be measured. In order to deduce the molecular implications of pesticides, the study is designed to expand the understanding of the impact of chlorpyrifos and dimethoate exposure on uterine (HOXA10, HOXA11, WNT5A) and ovarian (ESR2, RSPO2) genes in female Wistar rats. The reproductive and developmental toxicity of chlorpyrifos and dimethoate has been reported in both male and female rats. However, the genetic and epigenetic alterations induced by chlorpyrifos and dimethoate exposure on uterine (HOXA10, HOXA11, WNT5A) and ovarian (ESR2, RSPO2) genes has not been evaluated. Hence, the study will investigate the effect of chlorpyrifos and dimethoate exposure on the expression and methylation profile of uterine (HOXA10, HOXA11, WNT5A) and ovarian (ESR2, RSPO2) genes in female Wistar rats. Moreover, functional studies in the adult ovary have shown a fundamental requirement of WNT signaling for normal ovarian function and fertility. Therefore, identifying the epigenetic players which could influence the WNT signaling cascade

could unveil the effect of pesticide exposure on the critical and sensitive windows of female reproduction and fertility.

6. Methodology

Objective 1: To investigate the effect of chlorpyrifos and dimethoate exposure on expression of uterine (HOXA10, HOXA11, WNT5A) and ovarian (ESR2, RSPO2) genes in female Wistar rats.

Approach: Healthy adult female Wistar albino rats of 6 weeks of age will be procured and will be divided into three groups (control, chlorpyrifos exposed and dimethoate exposed) each group having five rats. After pesticide exposure for 21 days, rats will be euthanized by CO_2 asphyxiation and uterus and ovary will be dissected and the tissues will be screened for the expression of HOXA10, HOXA11, WNT5A, ESR2 and RSPO2 genes.

- Experimental animals
- Chlorpyrifos and dimethoate exposure
- Determination of estrous cycle
- Hormonal and biochemical assessment
- Ultrasonography
- Body and organ weights
- Histopathology
- Immunohistochemistry
- Gene expression studies
- Western blot

a) Experimental animals

Adult female Wistar rats (N=18) of 6 weeks age and 150-200 grams will be purchased from registered animal facility. Rats will be housed in the Laboratory Animal House,

Faculty of Veterinary Sciences, and Animal Husbandry, SKUAST- Kashmir, J and K. After acclimatization, rats will be divided into three different groups:

Group 1: Control group (N=6)

Group 2: Chlorpyrifos exposed group (N=6)

Group 3: Dimethoate exposed group (N=6)

b) Chlorpyrifos and dimethoate exposure

Control group will receive only 500ul of castor oil by oral intubation for 21 days. Experimental groups will be given chloropyrifos at 1 mg/kg/day for 21 days on daily basis by oral intubation (Group 2) and dimethoate at the dose of 7mg/kg/day for 21 days on daily basis by oral intubation (Group 3). The animals will be sacrificed on the completion of the experiment and the reproductive organs were collected, weighed and processed for analysis.

c) Determination of estrous cycle

Estrous cyclicity shall be monitored by daily observations of vaginal smears.

d) Hormonal and biochemical assessment

Hormonal measurement shall be done after deep anaesthesia from the abdominal aorta during the estrus phase. Serum samples will be analysed for testosterone (T), luteinizing hormone (LH), follicle-stimulating hormone (FSH), estrogen (E2), progesterone (P), Anti-Mullerian hormone (AMH) and sex hormone-binding globulin (SHBG) using specific ELISA kits. Serum total cholesterol, High Density Lipoprotein (HDL) Low Density Lipoprotein (LDL) cholesterol and triglycerides will be estimated using autoanalyser.

e) Ultrasonography

The ultrasonography assessment will be performed to evaluate size and shape of various reproductive organs.

f) Body and organ weights

Body weights of the animals will be recorded weekly during the study. Before sacrifice, animals will be weighed again and subsequently the ovaries and uterus will be excised and weights will be recorded.

g) Histopathology

Uterus and ovarian tissues will be collected and coded at sacrifice, fixed in 5% neutral buffered formaldehyde for 24h at 4°C/stored at 4°C in 70% EtOH and subsequently processed. For each animal, organs will be kept paraffin embedded and sliced into 5µm sections (at 100µm intervals). Sections will be slide mounted and two mid-level slides per animal will be deparaffinized, stained with hematoxylin and eosin, coverslipped and qualitatively examined for histological abnormalities.

h) Immunohistochemistry

Tissue sections shall be deparaffinized in Citrisolv and rehydrated in PBS for 10 min. Antigen retrieval shall be performed by microwaving slides in 0.01 M sodium citrate buffer (pH 6.0) for 15 min. The sections shall be blocked with 1% serum for 30 min and incubated with the primary antibodies at room temperature overnight in a humidified chamber. Biotinylated anti-rabbit or goat secondary antibodies shall be added and incubated for 60 min at room temperature. Detection shall be with streptavidin–FITC. All sections shall be counterstained with ethidium homodimer-2. After three washes in PBS, slides shall be mounted in Prolong Gold Anti-fade reagent. Negative controls sections shall be treated identical, except primary antibody will be replaced with buffer solution. Sections shall be analyzed and imaged using microscopically with epifluorescent attachments and suitable filters for streptavidin–FITC (green) and EthD-2 detection (red).

i) Gene expression studies

Expression of genes HOXA10, HOXA11, Wnt5a (uterine), ESR2 and RSPO2 (ovarian) will be carried out using qPCR. The expression of indicated genes will be normalized to the GAPDH mRNA level. Specificity of amplification will be confirmed by melting curve analysis.

j) Western blot

Tissues of uterus and ovaries will be homogenized and total protein will be extracted. Protein estimation will be done by Bradford method. Protein extracted from each sample will be electrophoresed in SDS polyacrylamide gels and then transferred to PVDF membranes. Membranes will be blocked with 5 % skim milk for 16–24 h and incubated overnight with diluted primary rat antibody for selected genes followed by a horse radish peroxidase (HRP)-conjugated secondary antibody against rat IgG. Bands with peroxidase activity will be detected by an enhanced chemiluminescent detection system and visualized using a G-Box chemiluminescence image capture system.

Objective 2: To identify the methylation profile of uterine (HOXA10, HOXA11, WNT5A) and ovarian (ESR2, RSPO2) genes following the exposure to chlorpyrifos and dimethoate.

Approach: In order to check the methylation status of uterine and ovarian genes following the chlorpyrifos and dimethoate exposure, methylation specific PCR will be done.

- Methylation specific PCR

DNA will be extracted from tissues of uterus and ovaries. DNA will be digested with methylation-sensitive and methylation-insensitive restriction enzymes overnight at 37^{o}C, followed by PCR using primer sets designed to amplify methylation sites. PCR

products will be separated using PAGE and visualized by SYBR green staining. Hypo or hypermethylation will be determined by the relative band intensity.

Objective 3: To investigate the molecular implications of chlorpyrifos and dimethoate exposure on regulation of WNT signaling in uterus and ovaries of female Wistar rats.

Approach: In order to determine the role of uterine and ovarian genes post chlorpyrifos and dimethoate exposure in regulating the WNT signaling, the expression of both canonical and non-canonical WNT genes will be determine by qPCR. Western blot will be done to check the protein expression.

- Gene expression studies
- Western blot

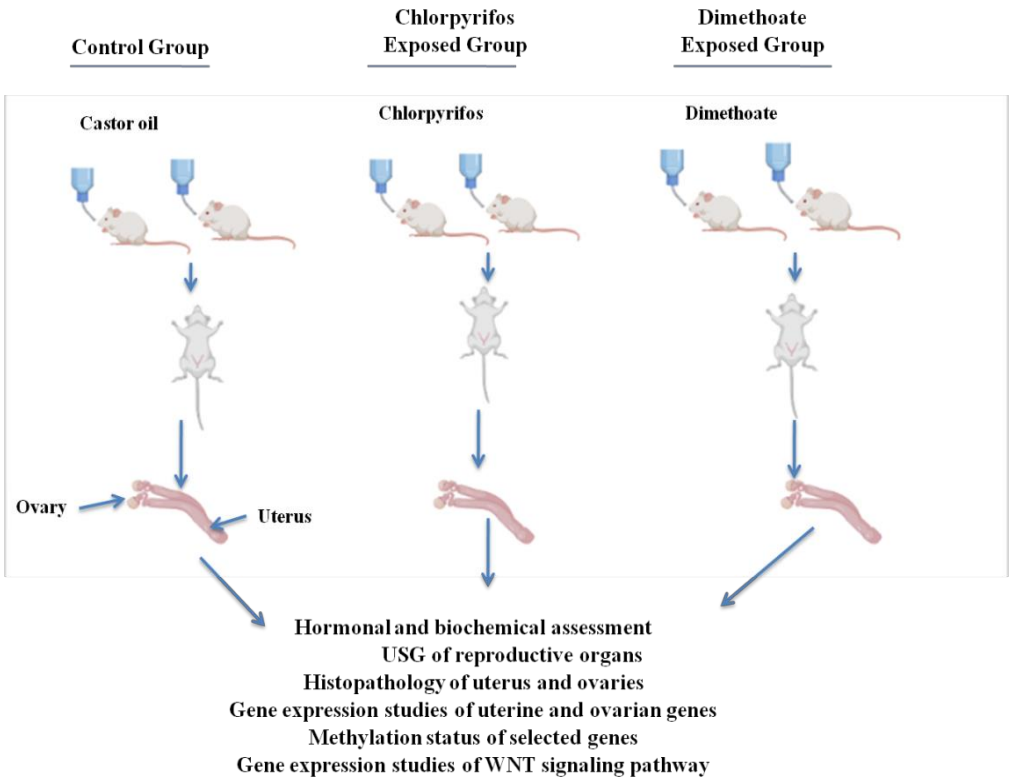
a) Gene expression studies

Expression of genes WNT1, WNT3a and β catenin (canonical), WNT5a and WNT11 (non-canonical) will be carried out using qPCR in both unexposed and exposed uterine and ovarian tissues. The expression of indicated genes will be normalized to the GAPDH mRNA level. Specificity of amplification will be confirmed by melting curve analysis.

b) Western blot

Tissues of uterus and ovaries (control and exposed) will be homogenized and total protein will be extracted. Protein estimation will be done by Bradford method. Protein extracted from each sample will be electrophoresed in SDS polyacrylamide gels and then transferred to PVDF membranes. Bands with peroxidase activity will be detected by an enhanced chemiluminescent detection system.

Overview of work plan



7. References

Abedini, A., Zamberlam, G., Boerboom, D., & Price, C. A. (2015). Non-canonical WNT5A is a potential regulator of granulosa cell function in cattle. *Molecular and Cellular Endocrinology*, *403*, 39-45.

Adigun, A. A., Wrench, N., Seidler, F. J., & Slotkin, T. A. (2010). Neonatal organophosphorus pesticide exposure alters the developmental trajectory of cell-signaling cascades controlling metabolism: differential effects of diazinon and parathion. *Environmental Health Perspectives*, *118*(2), 210-215.

Attia, A. A., ElMazoudy, R. H., & El-Shenawy, N. S. (2012). Antioxidant role of propolis extract against oxidative damage of testicular tissue induced by insecticide chlorpyrifos in rats. *Pesticide biochemistry and physiology*, *103*(2), 87-93.

Axelsson, J., Bonde, J. P., Giwercman, Y. L., Rylander, L., & Giwercman, A. (2010). Gene-environment interaction and male reproductive function. *Asian journal of andrology*, *12*(3), 298.

Azziz, R., Carmina, E., Chen, Z., Dunaif, A., Laven, J. S., Legro, R. S., ... & Yildiz, B. O. (2016). Polycystic ovary syndrome. *Nature reviews Disease primers*, *2*(1), 1-18.

Baig, S. A., Akhtera, N. A., Ashfaq, M., & Asi, M. R. (2009). Determination of the organophosphorus pesticide in vegetables by high-performance liquid chromatography. *American-Eurasian Journal of Agriculture and Environmental Science*, *6*(5), 513-519.

Bell, S. M., Schreiner, C. M., Hess, K. A., Anderson, K. P., & Scott, W. J. (2003). Asymmetric limb malformations in a new transgene insertional mutant, footless. *Mechanisms of development*, *120*(5), 597-605.

Blitek, A., Kiewisz, J., Waclawik, A., Kaczmarek, M. M., & Ziecik, A. J. (2010). Effect of steroids on HOXA10 mRNA and protein expression and prostaglandin production in the porcine endometrium. *Journal of Reproduction and Development*, 1008250298-1008250298.

Boerboom, D., Paquet, M., Hsieh, M., Liu, J., Jamin, S. P., Behringer, R. R., ... & Richards, J. S. (2005). Misregulated Wnt/β-catenin signaling leads to ovarian granulosa cell tumor development. *Cancer research*, *65*(20), 9206-9215.

Borahay, M. A., Asoglu, M. R., Mas, A., Adam, S., Kilic, G. S., & Al-Hendy, A. (2017). Estrogen receptors and signaling in fibroids: role in pathobiology and therapeutic implications. *Reproductive sciences*, *24*(9), 1235-1244.

Castañon, B. I., Stapp, A. D., Gifford, C. A., Spicer, L. J., Hallford, D. M., & Gifford, J. H. (2012). Follicle-stimulating hormone regulation of estradiol production:

possible involvement of WNT2 and β-catenin in bovine granulosa cells. *Journal of Animal Science*, *90*(11), 3789-3797.

Chassot, A. A., Ranc, F., Gregoire, E. P., Roepers-Gajadien, H. L., Taketo, M. M., Camerino, G., ... & Chaboissier, M. C. (2008). Activation of β-catenin signaling by Rspo1 controls differentiation of the mammalian ovary. *Human molecular genetics*, *17*(9), 1264-1277.

Chen, M., Wolfe, A., Wang, X., Chang, C., Yeh, S., & Radovick, S. (2009). Generation and characterization of a complete null estrogen receptor α mouse using Cre/LoxP technology. *Molecular and cellular biochemistry*, *321*(1), 145-153.

Chen, Y., Jefferson, W. N., Newbold, R. R., Padilla-Banks, E., & Pepling, M. E. (2007). Estradiol, progesterone, and genistein inhibit oocyte nest breakdown and primordial follicle assembly in the neonatal mouse ovary in vitro and in vivo. *Endocrinology*, *148*(8), 3580-3590.

Cheng, G., Weihua, Z., Mäkinen, S., Mäkelä, S., Saji, S., Warner, M., ... & Hovatta, O. (2002). A role for the androgen receptor in follicular atresia of estrogen receptor beta knockout mouse ovary. *Biology of reproduction*, *66*(1), 77-84.

Connell, M. T., Owen, C. M., & Segars, J. H. (2013). Genetic syndromes and genes involved in the development of the female reproductive tract: a possible role for gene therapy. *Journal of genetic syndromes & gene therapy*, *4*.

Cummings, S. R., Browner, W. S., Bauer, D., Stone, K., Ensrud, K., Jamal, S., & Ettinger, B. (1998). Endogenous hormones and the risk of hip and vertebral fractures among older women. *New England Journal of Medicine*, *339*(11), 733-738.

Dada, R., Kumar, M., Jesudasan, R., Fernández, J. L., Gosálvez, J., & Agarwal, A. (2012). Epigenetics and its role in male infertility. *Journal of assisted reproduction and genetics*, *29*(3), 213-223.

Daftary, G. S., & Taylor, H. S. (2006). Endocrine regulation of HOX genes. *Endocrine reviews*, *27*(4), 331-355.

Delbes, G., Blázquez, M., Fernandino, J. I., Grigorova, P., Hales, B. F., Metcalfe, C., ... & Marlatt, V. (2022). Effects of endocrine disrupting chemicals on gonad development: Mechanistic insights from fish and mammals. *Environmental Research*, *204*, 112040.

Depmann, M., Faddy, M. J., Van Der Schouw, Y. T., Peeters, P. H. M., Broer, S. L., Kelsey, T. W., ... & Broekmans, F. J. M. (2015). The relationship between variation in size of the primordial follicle pool and age at natural menopause. *The Journal of Clinical Endocrinology & Metabolism*, *100*(6), E845-E851.

Diamanti-Kandarakis, E., Bourguignon, J. P., Giudice, L. C., Hauser, R., Prins, G. S., Soto, A. M., ... & Gore, A. C. (2009). Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocrine reviews*, *30*(4), 293-342.

Du, H., & Taylor, H. S. (2016). The role of Hox genes in female reproductive tract development, adult function, and fertility. *Cold Spring Harbor perspectives in medicine*, *6*(1), a023002.

Dupont, S., Krust, A., Gansmuller, A., Dierich, A., Chambon, P., & Mark, M. (2000). Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. *Development*, *127*(19), 4277-4291.

Duursen, M.B.M.V., Boberg, J., Christiansen, S., Conolly, L., Damdimopoulou, P., Filis, P., Fowler, P.A., Gadella, B.M., Holte, J., Johansson, H.K.L., Mazaud-Guittot, S., Parent, A.S., Salumets, A., Soto, A.M., Svingen, T., Velthut-Meikas, A., Wedebye, E. B., Xie, Y., Berg, M.V.D. (2020). Safeguarding female reproductive health against endocrine disrupting chemicals-the FREIA project. Int. J. Mol. Sci. 21 (9), 3215.

El-Kashoury, A. A., & El-Din, H. T. (2010). Chlorpyrifos (from different sources): effect on testicular biochemistry of male albino rats. *J Am Sci*, *6*(7), 252-261.

EPA (US Environmental Protection Agency), 2008. EPA's 2008 Report on the Environment. National Center for Environmental Assessment, Washington, DC; EPA/600/R-07/045F. Available from the National Technical Information Service, Springfield, VA.

Farag, A. T., El-Aswad, A. F., & Shaaban, N. A. (2007). Assessment of reproductive toxicity of orally administered technical dimethoate in male mice. *Reproductive Toxicology*, *23*(2), 232-238.

Filippou, P., & Homburg, R. (2017). Is foetal hyperexposure to androgens a cause of PCOS?. *Human Reproduction Update*, *23*(4), 421-432.

Fowler, P. A., Bellingham, M., Sinclair, K. D., Evans, N. P., Pocar, P., Fischer, B., ... & O'Shaughnessy, P. J. (2012). Impact of endocrine-disrupting compounds (EDCs) on female reproductive health. *Molecular and cellular endocrinology*, *355*(2), 231-239.

Freytag, D., Günther, V., Maass, N., & Alkatout, I. (2021). Uterine fibroids and infertility. *Diagnostics*, *11*(8), 1455.

Giles, R. H., Van Es, J. H., & Clevers, H. (2003). Caught up in a Wnt storm: Wnt signaling in cancer. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer*, *1653*(1), 1-24.

Gold, L. S., Slone, T. H., Ames, B. N., & Manley, N. B. (2001). Pesticide residues in food and cancer risk: A critical analysis. *Handbook of pesticide toxicology*, *1*, 799-843.

Golezar, S., Ramezani Tehrani, F., Khazaei, S., Ebadi, A., & Keshavarz, Z. (2019). The global prevalence of primary ovarian insufficiency and early menopause: a meta-analysis. *Climacteric*, *22*(4), 403-411.

Grube, M., Neis, F., Brucker, S. Y., Kommoss, S., Andress, J., Weiss, M., ... & Krämer, B. (2019). Uterine Fibroids-Current Trends and Strategies. *Surgical Technology International*, *34*, 257-263.

Gupta, P. P., Folger, J. K., Rajput, S. K., Lv, L., Yao, J., Ireland, J. J., & Smith, G. W. (2014). Regulation and regulatory role of WNT signaling in potentiating FSH action during bovine dominant follicle selection. *PloS one*, *9*(6), e100201.

Harwood, B. N., Cross, S. K., Radford, E. E., Haac, B. E., & De Vries, W. N. (2008). Members of the WNT signaling pathways are widely expressed in mouse ovaries, oocytes, and cleavage stage embryos. *Developmental Dynamics*, *237*(4), 1099-1111.

Hewitt, S. C., O'Brien, J. E., Jameson, J. L., Kissling, G. E., & Korach, K. S. (2009). Selective disruption of ERα DNA-binding activity alters uterine responsiveness to estradiol. *Molecular endocrinology*, *23*(12), 2111-2116.

Hsieh, M., Johnson, M. A., Greenberg, N. M., & Richards, J. S. (2002). Regulated expression of Wnts and Frizzleds at specific stages of follicular development in the rodent ovary. *Endocrinology*, *143*(3), 898-908.

Jefferson, W. N., Chevalier, D. M., Phelps, J. Y., Cantor, A. M., Padilla-Banks, E., Newbold, R. R., ... & Williams, C. J. (2013). Persistently altered epigenetic marks in the mouse uterus after neonatal estrogen exposure. *Molecular endocrinology*, *27*(10), 1666-1677.

Jeon, G. H., Kim, S. H., Yun, S. C., Chae, H. D., Kim, C. H., & Kang, B. M. (2010). Association between serum estradiol level and coronary artery calcification in postmenopausal women. *Menopause*, *17*(5), 902-907.

Jirge, P. R. (2016). Poor ovarian reserve. *Journal of human reproductive sciences*, *9*(2), 63.

Johansson, Hanna K.L., Damdimopoulou, P., van Duursen, M.B.M., Boberg, J., Franssen, D., de Cock, M., J¨ a¨ ager, K., Wagner, M., Velthut-Meikas, A., Xie, Y., Connolly, L., Lelandais, P., Mazaud-Guittot, S., Salumets, A., Draskau, M.K., Filis, P., Fowler, P.A., Christiansen, S., Parent, A.S., Svingen, T. (2020). Putative adverse outcome pathways for female reproductive disorders to improve testing and regulation of chemicals. Arch. Toxicol. 94, 3359–3379.

Karabasanavar, N. S., & Singh, S. P. (2012). Occurrence of Chlorpyrifos Residues in Milk of Terai Region of Uttarakhand, India. *Indian Journal of Dairy Science*, *65*(5).

Kaur, S., & Dhanju, C. K. (2005). Biochemical effects of some organophosphorus pesticides on the ovaries of albino rats. *Indian journal of physiology and pharmacology*, *49*(2), 148.

Kenigsberg, S., Bentov, Y., Chalifa-Caspi, V., Potashnik, G., Ofir, R., & Birk, O. S. (2009). Gene expression microarray profiles of cumulus cells in lean and overweight-obese polycystic ovary syndrome patients. *Molecular human reproduction*, *15*(2), 89-103.

Kezele, P., & Skinner, M. K. (2003). Regulation of ovarian primordial follicle assembly and development by estrogen and progesterone: endocrine model of follicle assembly. *Endocrinology*, *144*(8), 3329-3337.

Kim, K. A., Kakitani, M., Zhao, J., Oshima, T., Tang, T., Binnerts, M., ... & Tomizuka, K. (2005). Mitogenic influence of human R-spondin1 on the intestinal epithelium. *Science*, *309*(5738), 1256-1259.

Komiya, Y., & Habas, R. (2008). Wnt signal transduction pathways. *Organogenesis*, *4*(2), 68-75.

Krege, J. H., Hodgin, J. B., Couse, J. F., Enmark, E., Warner, M., Mahler, J. F., ... & Smithies, O. (1998). Generation and reproductive phenotypes of mice lacking estrogen receptor β. *Proceedings of the National Academy of Sciences*, *95*(26), 15677-15682.

Liu, J., Wu, Q., Hao, Y., Jiao, M., Wang, X., Jiang, S., & Han, L. (2021). Measuring the global disease burden of polycystic ovary syndrome in 194 countries: Global Burden of Disease Study 2017. *Human Reproduction*, *36*(4), 1108-1119.

Logan, C. Y., & Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annu. Rev. Cell Dev. Biol.*, *20*, 781-810.

Louis, G. M. B., Chen, Z., Schisterman, E. F., Kim, S., Sweeney, A. M., Sundaram, R., ... & Barr, D. B. (2015). Perfluorochemicals and human semen quality: the LIFE study. *Environmental health perspectives*, *123*(1), 57-63.

Lubahn, D. B., Moyer, J. S., Golding, T. S., Couse, J. F., Korach, K. S., & Smithies, O. (1993). Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proceedings of the National Academy of Sciences*, *90*(23), 11162-11166.

Macon, M. B., & Fenton, S. E. (2013). Endocrine disruptors and the breast: early life effects and later life disease. *Journal of mammary gland biology and neoplasia*, *18*(1), 43-61.

Mahadevaswami, M. P., & Kaliwal, B. B. (2002). Effect of dimethoate administration schedules on compensatory ovarian hypertrophy, follicular dynamics, and estrous cycle in hemicastrated mice. *Journal of basic and clinical physiology and pharmacology*, *13*(3), 225-248.

Mancini, F., Van Bruggen, A. H., Jiggins, J. L., Ambatipudi, A. C., & Murphy, H. (2005). Acute pesticide poisoning among female and male cotton growers in India. *International journal of occupational and environmental health*, *11*(3), 221-232.

Marques-Pinto, A., & Carvalho, D. (2013). Human infertility: are endocrine disruptors to blame?. *Endocrine connections*, *2*(3), R15-R29.

Martín Reina, J., Duarte, J. A., Cerrillos, L., Bautista Palomas, J. D., & Moreno Navarro, I. M. (2017). Insecticide reproductive toxicity profile: organophosphate, carbamate and pyrethroids. *Journal of Toxins, 4 (1), 1-7*.

Mathur, H. B., Agarwal, H. C., Johnson, S., & Saikia, N. (2005). Analysis of pesticide residues in blood samples from villages of Punjab. *Centre for Science and Environment, New Delhi*, 1-24.

Miller, J. R., Hocking, A. M., Brown, J. D., & Moon, R. T. (1999). Mechanism and function of signal transduction by the Wnt/β-catenin and Wnt/Ca2+ pathways. *Oncogene*, *18*(55), 7860-7872.

Modi, D., & Godbole, G. (2009). HOXA10 signals on the highway through pregnancy. *Journal of reproductive immunology*, *83*(1-2), 72-78.

Morais, S., Dias, E., & Pereira, M. L. (2012). Carbamates: human exposure and health effects. *The impact of pesticides*, 21-38.

Mostafalou, S., & Abdollahi, M. (2013). Pesticides and human chronic diseases: evidences, mechanisms, and perspectives. *Toxicology and applied pharmacology*, *268*(2), 157-177.

Mostafalou, S., & Abdollahi, M. (2017). Pesticides: an update of human exposure and toxicity. *Archives of toxicology*, *91*(2), 549-599.

Mtango, N. R., Potireddy, S., & Latham, K. E. (2008). Oocyte quality and maternal control of development. *International review of cell and molecular biology*, *268*, 223-290.

Nam, J. S., Park, E., Turcotte, T. J., Palencia, S., Zhan, X., Lee, J., ... & Yoon, J. K. (2007). Mouse R-spondin2 is required for apical ectodermal ridge maintenance in the hindlimb. *Developmental biology*, *311*(1), 124-135.

Nassan, F. L., Chavarro, J. E., & Tanrikut, C. (2018). Diet and men's fertility: does diet affect sperm quality?. *Fertility and sterility*, *110*(4), 570-577.

Newbold, R. R., Padilla-Banks, E., & Jefferson, W. N. (2006). Adverse effects of the model environmental estrogen diethylstilbestrol are transmitted to subsequent generations. *Endocrinology*, *147*(6), s11-s17.

Ngoulaa, F., Watchob, P., Bousekob, T. S., Kenfacka, A., Tchoumbouéa, J., & Kamtchouing, P. (2007). Effects of propoxur on the reproductive system of male rats. *African Journal of Reproductive Health*, *11*(1), 125-132.

Okoroiwu, H. U., & Iwara, I. A. (2018). Dichlorvos toxicity: A public health perspective. *Interdisciplinary toxicology*, *11*(2), 129.

Parker, W. H. (2007). Etiology, symptomatology, and diagnosis of uterine myomas. *Fertility and sterility*, *87*(4), 725-736.

Parma, P., Radi, O., Vidal, V., Chaboissier, M. C., Dellambra, E., Valentini, S., ... & Camerino, G. (2006). R-spondin1 is essential in sex determination, skin differentiation and malignancy. *Nature genetics*, *38*(11), 1304-1309.

Plante, I., Winn, L. M., Vaillancourt, C., Grigorova, P., & Parent, L. (2022). Killing two birds with one stone: Pregnancy is a sensitive window for endocrine effects on both the mother and the fetus. *Environmental Research*, *205*, 112435.

Polakis, P. (2000). Wnt signaling and cancer. *Genes & development*, *14*(15), 1837-1851.

Rannevik, G., Jeppsson, S., Johnell, O. A., Bjerre, B., Laurell-Borulf, Y., & Svanberg, L. (1995). A longitudinal study of the perimenopausal transition: altered profiles of steroid and pituitary hormones, SHBG and bone mineral density. *Maturitas*, *21*(2), 103-113.

Richards, J. S., Sharma, S. C., Falender, A. E., & Lo, Y. H. (2002). Expression of FKHR, FKHRL1, and AFX genes in the rodent ovary: evidence for regulation by IGF-I, estrogen, and the gonadotropins. *Molecular endocrinology*, *16*(3), 580-599.

Richardson, M. C., Guo, M., Fauser, B. C. J. M., & Macklon, N. S. (2014). Environmental and developmental origins of ovarian reserve. *Human reproduction update*, *20*(3), 353-369.

Ricken*, A., Lochhead, P., Kontogiannea, M., & Farookhi, R. (2002). Wnt signaling in the ovary: identification and compartmentalized expression of wnt-2, wnt-2b, and frizzled-4 mRNAs. *Endocrinology*, *143*(7), 2741-2749.

Sanchez, A. M., Viganò, P., Quattrone, F., Pagliardini, L., Papaleo, E., Candiani, M., & Panina-Bordignon, P. (2014). The WNT/β-catenin signaling pathway and expression of survival promoting genes in luteinized granulosa cells: endometriosis as a paradigm for a dysregulated apoptosis pathway. *Fertility and sterility*, *101*(6), 1688-1696.

Sharma, A., Kumar, V., Shahzad, B., Tanveer, M., Sidhu, G. P. S., Handa, N., ... & Thukral, A. K. (2019). Worldwide pesticide usage and its impacts on ecosystem. *SN Applied Sciences*, *1*(11), 1-16.

Skinner, M. K. (2005). Regulation of primordial follicle assembly and development. *Human reproduction update*, *11*(5), 461-471.

Taylor, H. S. (2000). The role of HOX genes in the development and function of the female reproductive tract. In *Seminars in reproductive medicine* (Vol. 18, No. 01, pp. 081-090).

Tiemann, U. (2008). In vivo and in vitro effects of the organochlorine pesticides DDT, TCPM, methoxychlor, and lindane on the female reproductive tract of mammals: a review. *Reproductive Toxicology*, *25*(3), 316-326.

Uimari, O., Järvelä, I., & Ryynänen, M. (2011). Do symptomatic endometriosis and uterine fibroids appear together?. *Journal of human reproductive sciences*, *4*(1), 34.

Upson, K. (2020). Environmental risk factors for endometriosis: a critical evaluation of studies and recommendations from the epidemiologic perspective. *Current epidemiology reports*, *7*(3), 149-170.

Vainio, S., Heikkilä, M., Kispert, A., Chin, N., & McMahon, A. P. (1999). Female development in mammals is regulated by Wnt-4 signalling. *Nature*, *397*(6718), 405-409.

van der Horst, P. H., Wang, Y., van der Zee, M., Burger, C. W., & Blok, L. J. (2012). Interaction between sex hormones and WNT/β-catenin signal transduction in endometrial physiology and disease. *Molecular and cellular endocrinology*, *358*(2), 176-184.

van der Stege, J. G., Groen, H., van Zadelhoff, S. J., Lambalk, C. B., Braat, D. D., van Kasteren, Y. M., ... & Hoek, A. (2008). Decreased androgen concentrations and diminished general and sexual well-being in women with premature ovarian failure. *Menopause*, *15*(1), 23-31.

Wang, H. X., Tekpetey, F. R., & Kidder, G. M. (2009). Identification of Wnt/β-catenin signaling pathway components in human cumulus cells. *Molecular human reproduction*, *15*(1), 11-17.

Wang, K., Yang, K., Xu, Q., Liu, Y., Li, W., Bai, Y., ... & Fang, M. (2019). Protein expression profiles in Meishan and Duroc sows during mid-gestation reveal differences affecting uterine capacity, endometrial receptivity, and the maternal–fetal Interface. *BMC genomics*, *20*(1), 1-11.

Wintermantel, T. M., Campbell, R. E., Porteous, R., Bock, D., Gröne, H. J., Todman, M. G., ... & Herbison, A. E. (2006). Definition of estrogen receptor pathway

critical for estrogen positive feedback to gonadotropin-releasing hormone neurons and fertility. *Neuron*, *52*(2), 271-280.

Zama, A. M., & Uzumcu, M. (2013). Targeted genome-wide methylation and gene expression analyses reveal signaling pathways involved in ovarian dysfunction after developmental EDC exposure in rats. *Biology of reproduction*, *88*(2), 52-1.

Zoeller, R. T., Brown, T. R., Doan, L. L., Gore, A. C., Skakkebaek, N. E., Soto, A. M., ... & Vom Saal, F. S. (2012). Endocrine-disrupting chemicals and public health protection: a statement of principles from The Endocrine Society. *Endocrinology*, *153*(9), 4097-4110.